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March 20, 2003

TSCA §8(e)  
US EPA  
1201 Constitution Ave. NW  
Room 3166  
Washington, DC 20460-0001



Re: Process Wash Solution Case 8EHQ-02-15192

To Whom It May Concern:

This letter contains the results of the study titled "6-Maleimidocaproic Acid – Acute Toxicity to *Daphnia Magna* Under Static Conditions," which is one of the components in the process wash solution previously submitted under the TSCA §8(e) notification referenced above. The 48-hour EC50 of this compound (CAS # 55750-53-3) was determined to be 772 mg/L and the No-Observed-Effect-Concentration (NOEC) was determined to be 451 mg/L. When compared with the study previously submitted, these data strongly suggest that the agent responsible for the effects reported therein were not due to 6-maleimidocaproic acid. Should you wish to discuss this in greater detail, please do not hesitate to contact me the number shown above.

Sincerely,

COMPANY SANITIZER

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**Study Title**

6-Maleimidocaproic Acid – Acute Toxicity to  
*Daphnia magna* Under Static Conditions

**Author**

Mark W. Machado

**Study Completed On**

29 January 2003

**Submitted To**

**Performing Laboratory**

Springborn Smithers Laboratories  
790 Main Street  
Wareham, Massachusetts 02571-1075

**Laboratory Project ID**

Springborn Smithers Study No. 13729.6102

**SUMMARY REPORT**

**SUMMARY****6-Maleimidocaproic Acid - Acute Toxicity to  
*Daphnia magna* Under Static Conditions****SPONSOR:****GUIDELINE:**

OECD Guideline # 202, *Daphnia* sp. Acute Immobilization Test and Reproduction Test. Adopted 4 April 1984.

**SPRINGBORN SMITHERS  
STUDY NUMBER:**

13729.6102

**TEST SUBSTANCE:**

6-Maleimidocaproic acid, a solid test substance, Batch No. 90224240, CAS# 55750-53-3, with a purity of 98.5%, was received

on 23 September 2002.

**EXPERIMENTAL  
TEST DATES:**

Preliminary Exposure #1: 2 to 4 October 2002

Preliminary Exposure #2: 13 to 15 November 2002

Preliminary Exposures #3 and #4: 3 to 5 December 2002

Definitive Exposure: 5 to 7 December 2002

**TEST VESSEL SIZE:**

250-mL glass beakers

**SOLUTION VOLUME:**

200 mL

**TEST ORGANISM:**

*Daphnia magna*

Source: Springborn Smithers Laboratories culture facility

**NUMBER OF TEST  
ORGANISMS PER VESSEL:**

5

**NUMBER OF TEST  
VESSELS PER TREATMENT:**

Preliminary Exposures # 1 and #2, and Definitive Exposure: 4  
Preliminary Exposures #3 and #4: 2

**DILUTION WATER:**

Fortified well water

pH: 7.9

Specific conductivity: 480 µmhos/cm

Total hardness as CaCO<sub>3</sub>: 170 mg/L

Total alkalinity as CaCO<sub>3</sub>: 110 mg/L

Total Organic Carbon (TOC): 0.60 mg/L for December 2002

**TEST CONDITIONS:**

48-hour duration, temperature range of 19 to 20°C, a photoperiod of 16 hours light: 8 hours darkness at a light intensity of 50 to 70 footcandles.

**NOMINAL TEST  
CONCENTRATIONS:**

Preliminary Exposure # 1: 0.063, 0.13, 0.25, 0.50 and 1.0 mg/L  
Preliminary Exposure #2: 250, 500, 1000, 2500 and 5000 mg/L  
Preliminary Exposures #3 and #4: 0.10, 1.0, 5.0, 10, 50 and 100 mg/L

Definitive Exposure: 100, 500, 1000, 2500 and 5000 mg/L

Note: The nominal test concentrations for the definitive exposure were prepared by dilution of a water-accommodated fraction having a concentration of 5.0 mg/mL. The dilution water was warmed to facilitate solubilization of the test substance and was mixed for approximately two hours with a magnetic stir bar and stir plate. The resultant solution was clear and yellow in color with a measured pH of 3.5. The pH was adjusted to 7.8 by adding drops of 5 M NaOH

**RESULTS:**

Based on extrapolated measured concentrations, the 48-hour EC50 was determined to be 772 mg/L with corresponding 95% confidence intervals of 452 to 902 mg/L. The No-Observed-Effect Concentration (NOEC) was determined to be 451 mg/L.

**Table 1. Preliminary Exposure #1: The pH, dissolved oxygen concentration and temperature measurements recorded during the 48-hour static acute exposure of *Daphnia magna* to 6-maleimidocaproic acid.**

Nominal Concentration (mg/L)	0 Hour	24 Hour	48-Hour
pH			
Control	7.8	7.8	7.8
0.063	7.8	7.8	7.8
0.13	7.8	7.8	7.8
0.25	7.8	7.8	7.8
0.50	7.7	7.8	7.8
1.0	7.7	7.8	7.8
Dissolved Oxygen, mg/L <sup>a</sup>			
Control	8.9	8.9	8.7
0.063	9.0	9.0	8.7
0.13	9.0	9.0	8.8
0.25	9.0	9.0	8.7
0.50	8.9	9.0	8.8
1.0	9.0	9.0	8.8
Temperature (°C) <sup>b</sup>			
	21	21	21

<sup>a</sup> 60% of saturation is 5.3 mg/L at 21°C.

<sup>b</sup> Values presented represent the daily temperatures measured (ERTCO brand thermometer) in replicate A of all test concentrations and the control at the stated time interval. Continuous temperature monitoring (Fisher Scientific min-max thermometer) in replicate D of the control test vessel established a temperature range of 19 to 22°C throughout the exposure period.

**Table 2. Preliminary Exposure #2: The pH, dissolved oxygen concentration and temperature measurements recorded during the 48-hour static acute exposure of *Daphnia magna* to 6-maleimidocaproic acid.**

Nominal Concentration (mg/L)	0 Hour	24 Hour	48-Hour
<b>pH</b>			
Control	7.8	8.0	7.6
250	6.1	6.9	NM <sup>c</sup>
500	5.2	5.8	NM
1000	4.4	4.5	NM
2500	3.8	3.6	NM
5000	3.5	3.6	NM
<b>Dissolved Oxygen, mg/L<sup>a</sup></b>			
Control	9.5	9.0	8.5
250	9.4	9.1	NM
500	9.2	9.0	NM
1000	9.1	9.1	NM
2500	9.1	9.1	NM
5000	9.1	9.1	NM
<b>Temperature (°C)<sup>b</sup></b>			
	20	20	20

<sup>a</sup> 60% of saturation is 5.4 mg/L at 20°C.

<sup>b</sup> Values presented represent the daily temperatures measured (Fisher brand thermometer) in replicate A of all test concentrations and the control at the stated time interval. Continuous temperature monitoring (Fisher Scientific min-max thermometer) in replicate D of the control test vessel established a temperature of 20°C throughout the exposure period.

<sup>c</sup> NM = Not measured due to 100% immobilization at the previous observation interval.

**Table 3. Preliminary Exposures #3 and #4: The pH, dissolved oxygen concentration and temperature measurements recorded during the 48-hour static acute exposure of *Daphnia magna* to 6-maleimidocaproic acid.**

Nominal Concentration (mg/L)	0 Hour	24 Hour	48-Hour
pH			
Control	7.8/7.8	7.8/7.8	7.8/7.8
0.1	7.8/7.8	7.8/7.8	7.8/7.9
1.0	7.8/7.8	7.8/7.8	7.8/7.9
5.0	7.8/7.8	7.8/7.8	7.8/7.9
10	7.8/7.8	7.7/7.7	7.8/7.9
50	7.6/7.6	7.4/7.5	7.6/7.6
100	7.2/7.6	7.2/7.3	7.3/7.4
Dissolved Oxygen, mg/L <sup>a</sup>			
Control	9.2/9.3	8.7/8.7	9.0/9.0
0.1	9.3/9.2	8.7/8.8	9.0/8.9
1.0	9.3/9.3	8.7/8.7	9.0/9.0
5.0	9.2/9.3	8.7/8.7	8.9/8.8
10	9.0/9.1	8.7/8.6	8.8/8.6
50	8.3/8.2	8.6/8.4	8.8/8.3
100	7.0/6.9	8.5/8.2	8.7/8.0
Temperature (°C) <sup>b</sup>			
	20/20	20/20	20/20

<sup>a</sup> 60% of saturation is 5.4 mg/L at 20°C.

<sup>b</sup> Values presented represent the daily temperatures measured (Fisher brand thermometer) in replicate A (for Exposure #3) and replicate C (for Exposure #4) of all test concentrations and the control at the stated time interval. Continuous temperature monitoring (Fisher Scientific min-max thermometer) in replicate D of the control test vessel established a temperature of 20°C throughout the exposure period.

**Table 4. Definitive Exposure: The pH, dissolved oxygen concentration and temperature measurements recorded during the 48-hour static acute exposure of *Daphnia magna* to 6-maleimidocaproic acid.**

Nominal Concentration (mg/L)	0 Hour	24 Hour	48-Hour
<b>pH</b>			
Control	7.7	7.7	7.8
100	7.7	7.7	7.8
500	7.7	7.7	7.7
1000	7.7	7.5	7.6
2500	7.7	7.3	NM <sup>c</sup>
5000	7.7	7.0	NM
<b>Dissolved Oxygen, mg/L<sup>a</sup></b>			
Control	9.2	8.7	8.9
100	9.1	8.6	8.8
500	9.0	8.7	8.7
1000	8.9	8.7	8.6
2500	8.2	8.7	NM
5000	6.9	8.6	NM
<b>Temperature (°C)<sup>b</sup></b>			
	21	20	20

<sup>a</sup> 60% of saturation is 5.4 mg/L at 20°C and 5.3 at 21°C.

<sup>b</sup> Values presented represent the daily temperatures measured (Fisher brand thermometer) in replicate A of all test concentrations and the control at the stated time interval. Continuous temperature monitoring (Fisher Scientific min-max thermometer) in replicate D of the control test vessel established a temperature range of 19 to 20°C throughout the exposure period.

<sup>c</sup> NM = Not measured due to 100% immobilization at the previous observation interval.



**Table 5. Preliminary Exposure #1: Nominal concentrations tested, corresponding cumulative number and percent immobilization, and observations made during the 48-hour static acute exposure of *Daphnia magna* to 6-maleimidocaproic acid.**

Nominal Concentration (mg/L)	Cumulative Percent of Immobilized Organisms <sup>a</sup>									
	24 Hour					48 Hour				
	A	B	C	D	Mean	A	B	C	D	Mean
Control	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
0.063	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
0.13	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
0.25	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
0.50	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
1.0	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0

Note: Stock solution mixed vigorously for 2.5 hours with a laboratory stirrer. Undissolved test substance was evident but removed during exposure solution preparation. pH of stock solution not adjusted.

<sup>a</sup> The actual number of immobilized organisms is presented in parentheses.

**Table 6. Preliminary Exposure #2: Nominal concentrations tested, corresponding cumulative number and percent immobilization, and observations made during the 48-hour static acute exposure of *Daphnia magna* to 6-maleimidocaproic acid.**

Nominal Concentration (mg/L)	Cumulative Percent of Immobilized Organisms <sup>a</sup>									
	24 Hour					48 Hour				
	A	B	C	D	Mean	A	B	C	D	Mean
Control	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
250	100 (5)	100 (5)	100 (5)	100 (5)	100	100 (5)	100 (5)	100 (5)	100 (5)	100
500	100 (5)	100 (5)	100 (5)	100 (5)	100	100 (5)	100 (5)	100 (5)	100 (5)	100
1000	100 (5)	100 (5)	100 (5)	100 (5)	100	100 (5)	100 (5)	100 (5)	100 (5)	100
2500 <sup>b</sup>	100 (5)	100 (5)	100 (5)	100 (5)	100	100 (5)	100 (5)	100 (5)	100 (5)	100
5000 <sup>c</sup>	100 (5)	100 (5)	100 (5)	100 (5)	100	100 (5)	100 (5)	100 (5)	100 (5)	100

Note: Stock solution heated to approximately 90°C and mixed vigorously for 8.0 hours with a laboratory stirrer. A very small amount of undissolved test substance was evident but removed during exposure solution preparation. The stock solution was observed to be slightly yellow in color. The pH of the stock solution was not adjusted. In addition, a sample of the stock solution was sent to the Study Sponsor for confirmation of test substance concentration. The results indicate a recovery of 4510 mg/L.

<sup>a</sup> The actual number of immobilized organisms is presented in parentheses.

<sup>b</sup> Solution very slightly yellow in color.

<sup>c</sup> Solution slightly yellow in color.

**Table 7. Preliminary Exposure #3: Nominal concentrations tested, corresponding cumulative number and percent immobilization, and observations made during the 48-hour static acute exposure of *Daphnia magna* to 6-maleimidocaproic acid.**

Nominal Concentration (mg/L)	Cumulative Percent of Immobilized Organisms <sup>a</sup>					
	24 Hour			48 Hour		
	A	B	Mean	A	B	Mean
Control	0 (0)	0 (0)	0	0 (0)	0 (0)	0
0.10	0 (0)	0 (0)	0	0 (0)	0 (0)	0
1.0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
5.0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
10	0 (0)	0 (0)	0	0 (0)	0 (0)	0
50	0 (0)	0 (0)	0	0 (0)	0 (0)	0
100	0 (0)	0 (0)	0	60 (3)	20 (1)	40 <sup>b</sup>

Note: Stock solution heated to approximately 90°C and mixed vigorously for 0.5 hours with a laboratory stirrer. The stock solution was observed to be clear and colorless. The pH of the stock solution was not adjusted.

<sup>a</sup> The actual number of immobilized organisms is presented in parentheses.

<sup>b</sup> All surviving daphnids were observed to be lethargic.

**Table 8. Preliminary Exposure #4: Nominal concentrations tested, corresponding cumulative number and percent immobilization, and observations made during the 48-hour static acute exposure of *Daphnia magna* to 6-maleimidocaproic acid.**

Nominal Concentration (mg/L)	Cumulative Percent of Immobilized Organisms <sup>a</sup>					
	24 Hour			48 Hour		
	C	D	Mean	C	D	Mean
Control	0 (0)	0 (0)	0	0 (0)	0 (0)	0
0.10	0 (0)	0 (0)	0	0 (0)	0 (0)	0
1.0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
5.0	0 (0)	0 (0)	0	0 (0)	0 (0)	0
10	0 (0)	0 (0)	0	0 (0)	0 (0)	0
50	0 (0)	0 (0)	0	0 (0)	0 (0)	0
100	0 (0)	0 (0)	0	0 (0)	0 (0)	0

Note: Stock solution heated to approximately 90°C and mixed vigorously for 0.5 hours with a laboratory stirrer. The stock solution was observed to be clear and colorless. The pH of the stock solution was adjusted from 7.2 to 7.8.

<sup>a</sup> The actual number of immobilized organisms is presented in parentheses.

**Table 9. Definitive Exposure: Nominal concentrations tested, corresponding cumulative number and percent immobilization, and observations made during the 48-hour static acute exposure of *Daphnia magna* to 6-maleimidocaproic acid.**

Nominal Concentration (mg/L)	Cumulative Percent of Immobilized Organisms <sup>a</sup>									
	24 Hour					48 Hour				
	A	B	C	D	Mean	A	B	C	D	Mean
Control	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
100	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
500	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)	0
1000	0 (0)	0 (0)	0 (0)	0 (0)	0 <sup>b</sup>	80 (4)	20 (1)	100 (5)	80 (4)	70 <sup>b</sup>
2500	100 (5)	100 (5)	100 (5)	100 (5)	100	100 (5)	100 (5)	100 (5)	100 (5)	100
5000	100 (5)	100 (5)	100 (5)	100 (5)	100	100 (5)	100 (5)	100 (5)	100 (5)	100

Note: Stock solution heated to approximately 90°C and mixed vigorously for 2.0 hours with a laboratory stirrer. The stock solution was observed to be clear and yellow in color. The pH of the stock solution was adjusted from 3.5 to 7.8.

<sup>a</sup> The actual number of immobilized organisms is presented in parentheses.

<sup>b</sup> Surviving daphnids were observed to be lethargic.

**Table 10. Definitive Exposure:** The EC50 values (corresponding 95% confidence intervals) and No-Observed-Effect Concentration (NOEC) for 6-maleimidocaproic acid and *Daphnia magna* under static conditions.

	EC50 (mg/L)	95% Confidence Intervals	
		Lower (mg/L)	Upper (mg/L)
24 Hour <sup>a</sup>	1430 <sup>b</sup>	902	2260
48 Hour <sup>a</sup>	772	452	902
NOEC through 48 hours = 451 mg/L			

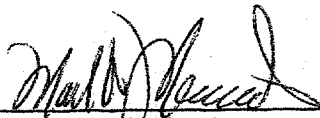
<sup>a</sup> EC50 values estimated by non-linear interpolation. Corresponding 95% confidence intervals calculated by binomial probability.

<sup>b</sup> Values presented are based on an extrapolation of measured concentrations of the stock solution during preliminary exposure #2.

**SIGNATURES AND APPROVAL**

**SUBMITTED BY:**

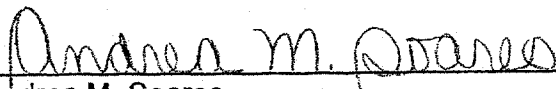
Springborn Smithers Laboratories  
790 Main Street  
Wareham, Massachusetts 02571-1075



Mark W. Machado  
Study Director

1/29/03

Date



Andrea M. Soares  
Technical Report Writer

1/29/03

Date



Ronald C. Biever  
Director, Environmental Toxicology

1-29-03

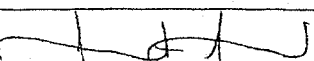
Date

## STUDY PROTOCOL



# TEST PROTOCOL

**PROTOCOL TITLE: Acute Toxicity to Water Fleas, (*Daphnia magna*) Under Static Conditions, Following OECD Guideline #202.**

TO BE COMPLETED BY THE STUDY SPONSOR:	
Study Sponsor:	
Address:	
Sponsor Protocol/Project No.:	
Test Substance Name(s):	
Purity: 98.5%	Batch or Lot #: 90224240
Analytical Standard N/A	
Purity: NA	Batch or Lot #: NA
Additional Comments and Modifications: NA	
Sponsor Approval: 	
Date: 09/25/02	

TO BE COMPLETED BY SPRINGBORN SMITHERS LABORATORIES BEFORE EXPERIMENT INITIATION:

Testing Facility: Springborn Smithers Laboratories 790 Main Street, Wareham, MA 02571-1075

Study Director: MARK W. MATHIAS

Study No.: 13729-6102

Test Concentration: 5000 2500 1000 500 100 mg/L plus control

Proposed Experimental Dates: 12/5/12 (Start) 12/7/12 (Termination)

  
Study Director Signature

10/1/62  
Study Initiation Date

**Acute Toxicity to Water Fleas, (*Daphnia magna*) Under Static Conditions, Following  
OECD Guideline #202.**

**1.0 OBJECTIVE**

The purpose of this test will be to determine the acute effects of a test substance on the water flea, *Daphnia magna*, under static conditions. Test results will be reported as 48-hour EC50 values, i.e., the median concentration that immobilizes 50% of the number of daphnids exposed, with 95% confidence limits. The methods described in this protocol generally meet the testing requirements of the Organization for Economic Co-operation and Development, OECD Guideline For Testing Of Chemicals #202, *Daphnia* sp. Acute Immobilization Test and Reproduction Test (OECD, 1984).

**2.0 MATERIALS AND METHODS**

**2.1 Chemical System**

**2.1.1 Test Substance**

Upon arrival at Springborn Smithers Laboratories, the test and reference substance(s) will be received by the Test Material Center. Records will be maintained in accordance with GLP requirements, and a Chain-of-Custody established. The condition of the external packaging of the test substance will be recorded and any damage noted. The packaging will be removed, the primary storage container inspected for leakage or damage, and the condition recorded. Any damage will be reported to the Sponsor and/or manufacturer.

Each sample will be given a unique sample ID number and stored under the conditions specified by the Sponsor or manufacturer. The following information should be provided by the Study Sponsor, if applicable: test substance lot or batch number, test substance purity, water solubility (pH and temperature of solubility determination), vapor pressure, storage stability, methods of analysis of the test substance in water, MSDS, and safe handling procedures, and a verified expiration or reanalysis date.

**2.1.2 Test Substance Concentration Selection**

Test substance concentrations will be based on the results of a preliminary range-finding test. The range of concentrations selected for the definitive test is intended to include both 100 and 0% immobilization, but due to the nature of some test substances, one or both levels may not be observed. If possible, the lowest concentration tested should preferably result in no observable effect in comparison to the control organisms. No attempt will be made to determine the degree of adsorption of the test substance by the test system, as this falls outside the scope and intent of this study. At least five concentrations and a negative control will be used. Each test substance concentration will differ by a constant factor not exceeding 2.0. A negative control consists of dilution water without the test substance.

### **2.1.3 Solvent Control**

An organic solvent may be used as a carrier to solubilize the test substance. In such a case, a solvent control will be included in the test, and consists of dilution water plus the highest concentration of solvent that occurs in any of the test solutions. The solvent concentration will be kept as low as possible, and will not be allowed to exceed 0.1 mL/L.

### **2.1.4 Stock Solution Preparation**

The test substance will be weighed on an analytical balance for which a calibration log will be maintained. A Chemical Usage Log will also be maintained in which the amount, the date, the intended use and the user's initials will be recorded each time the test substance is used. A dosing stock solution will be prepared by dissolving an appropriate amount of test substance in carrier (distilled water or solvent). The stock solution will be stored in properly labeled containers until needed.

### **2.1.5 Exposure Solution Preparation**

Each test concentration of test substance will be prepared by adding an appropriate aliquot of the stock solution directly into 1.0-L of dilution water in an intermediate vessel and mixing thoroughly. Each respective test solution (1.0-L) will then be divided between four replicate test vessels, with each test vessel receiving 200-mL of test solution.

## **2.2 Test System**

### **2.2.1 Species**

The water flea, *Daphnia magna*, will be the species used in this test. Test organisms will be  $\leq 24$  hours old at the initiation of the test. Daphnids will be obtained by removing all immature daphnids from the culture vessel, thus isolating sexually mature daphnids  $\leq 24$  hours prior to initiating the test. Young produced by these organisms will be subsequently pipetted into the test beakers.

### **2.2.2 Justification of Test System**

Characteristics which make this test organism suitable for this acute toxicity test are their ease of handling and their sensitivity to a variety of chemical substances, and the extensive data base for this common freshwater invertebrate species.

### **2.2.3 Origin**

*Daphnia magna* cultures will be maintained at Springborn Smithers Laboratories. Daphnids will be cultured in 1.0-L glass vessels containing 0.80 L of water. Water used to culture the daphnids will be prepared in the same manner and have the same characteristics as described for dilution water. Culture water will be maintained at  $20 \pm 2^\circ\text{C}$ . Each culture vessel will be cleaned at least once weekly.

#### **2.2.4 Feeding**

While being maintained in culture prior to the test, organisms will be fed daily 1.0 mL of a unicellular green algae, *Ankistrodesmus falcatus* and 0.5 mL of a combination of yeast, cereal leaves and flaked fish food (YCT). The food solution will be prepared to contain approximately  $4 \times 10^7$  cells/mL of algae. Daphnids will not be fed during the 48-hour exposure period. Samples of each food source will be periodically analyzed for the presence of pesticides, PCBs and selected toxic metals.

#### **2.2.5 Handling**

Wide-bore pipets will be used to transfer the daphnids, taking care to minimize possible stress due to handling. Daphnids that are damaged or dropped during transfer will not be used.

#### **2.2.6 Loading**

At least 2 ml of the test solution should be provided for each animal.

### **2.3 Physical System**

#### **2.3.1 Test Vessels**

Test vessels will be 250-mL glass beakers. The test vessels will be chemically cleaned before the test is started. The test vessels will be washed with hot water and a detergent, rinsed with acetone, and then rinsed extensively with water. The total test solution volume in each test vessel will be 200-mL. Test vessels will be labeled to identify the study number, treatment/control and the replicate designation.

#### **2.3.2 Replication and Control of Bias**

Four replicates will be included with each test concentration and control. Each replicate vessel will contain five individuals, a total of 20 daphnids per concentration or control. Daphnids will be added impartially to each test jar by adding no more than two daphnids to each vessel until all jars contain two daphnids. This procedure will be repeated until each vessel contains five daphnids.

#### **2.3.3 Dilution Water**

Dilution water will consist of hard fortified well water with a total hardness of 160 to 180 mg/L as  $\text{CaCO}_3$ . The well water (total hardness about 30 to 60 mg/L as  $\text{CaCO}_3$ ) will be fortified according to the formulation for hard water presented in "Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians" (U.S. EPA, 1975). Dilution water will be filtered through an amberlite XAD-7 resin column. The column is 30 cm long and 1.6 cm wide. This filtration will effectively remove any potential organic contaminants from the water. Total hardness, total alkalinity, pH and specific conductance of the diluent water will be monitored on each batch prior to use to assure that these parameters are within the normal acceptable ranges. Total hardness and alkalinity will be determined according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 1992). Ranges for these parameters will generally be:

total hardness, 160 to 180 mg/L as  $\text{CaCO}_3$ ; alkalinity, 110 to 130 mg/L as  $\text{CaCO}_3$ ; specific conductance, 400 to 600  $\mu\text{mhos/cm}$ ; and pH, 7.9 to 8.3.

Quality of the dilution water used to conduct daphnid acute tests will be judged by the ability of the daphnid cultures to survive and reproduce in the water free of stress. The dilution water will be prepared in 1,900-L batches. New batches of diluent water will be prepared when either the previous batch is exhausted, when a water quality parameter (total hardness, alkalinity, etc.) differs from the normal ranges, or after two weeks of holding. The diluent water will be aerated with an air pump and air stones to bring the pH and dissolved gases into equilibrium with the atmosphere. Fiberglass containers will be used to hold the diluent water. Periodic analysis of representative samples of the dilution water source will be conducted to ensure the absence of potential toxicants, including pesticides, PCBs and selected toxic metals, at concentrations which may be harmful to daphnids.

## **2.4 Test Conditions**

### **2.4.1 Temperature**

Water temperature of the test solutions will be maintained at  $20 \pm 1^\circ\text{C}$  by conducting the test in a temperature-controlled room or water bath maintained at the appropriate test temperature.

### **2.4.2 Lighting**

The test will be conducted in a light controlled laboratory. The test will be illuminated to a light intensity of 30 to 100 footcandles using fluorescent bulbs. Light intensity around the test area will be measured and recorded once during the study. A 16-hour light, 8-hour dark photoperiod will be maintained with an automatic timer.

### **2.4.3 Dissolved Oxygen**

Total dissolved oxygen will not be allowed to drop below 60% of saturation for the duration of the test.

### **2.4.4 Test Initiation**

The test begins when all the daphnids have been impartially placed in the test vessels and terminates after 48 hours of exposure.

## **2.5 Sampling and Observations**

### **2.5.1 Water Quality Measurements**

At test initiation and daily thereafter, water quality variables (temperature, pH and dissolved oxygen concentrations) will be measured and recorded in one representative test vessel (replicate A) of each concentration and control. If 100% mortality is observed in any test vessel, water quality measurements will be discontinued. Measurement techniques will follow methods described in *Standard Methods for the Examination of Water and Wastewater* (APHA, 1992). The temperature range will be monitored

continuously in one test solution by using a minimum-maximum thermometer. Readings of temperature extremes will be recorded daily.

#### **2.5.2 Biological Observations**

The number of immobilized daphnids in each test vessel will be recorded after 24 and 48 hours of test initiation. Immobilization is defined as those animals not able to swim within 15 seconds after gentle agitation of the test container. The test will be terminated following 48 hours of exposure. In addition, whenever test organisms are observed, characteristics of the test solutions will also be observed and recorded, e.g., precipitated materials, cloudiness, etc.

#### **2.5.3 Acceptability Criteria**

During the definitive test, in the controls (either negative or solvent control), not more than 10% of the daphnids at test termination should be immobilized or trapped on the surface of the water or the test will be considered unacceptable.

### **3.0 STATISTICAL ANALYSES**

Test results derived from the acute test will be used to statistically estimate a median effective concentration (EC50) and its 95% confidence interval after 24 and 48 hours of exposure. The EC50 is the estimated measured concentration of the test substance in dilution water which produces 50% immobilization in the test populations of daphnids at the stated times of exposure. EC50 values will be computed using mean measured concentrations.

A computer program will be used to estimate EC50 values using three statistical methods: probit analysis, moving average method, and binomial probability. The method selected and reported will be determined by the data base (i.e., presence or absence of 100% response, number of partial responses, etc.). An EC50 value cannot be calculated if the data derived is insufficient according to any of the three statistical methods. The probit method provides values of the slope, including 95% confidence intervals, as well as appropriate statistical tests to evaluate goodness-of-fit. In addition, if possible, the highest concentration tested that produced no immobile daphnids and the lowest concentration tested that produced 100% immobilized daphnids will be reported.

### **4.0 RECORDS TO BE MAINTAINED**

Records to be maintained will include, but will not be limited to, correspondence and other documents relating to the interpretation and evaluation of data as well as all raw data and documentation generated as a result of the study.

### 5.0 REPORTING

The raw data and the study report will be reviewed by the Study Director. All values will be reported to various levels of significance depending on the accuracy of the measuring devices employed during any one process.

### 6.0 SPECIAL PROVISIONS

**TEST SUBSTANCE DISPOSAL:** After the issuance of the final report, the test substance will be returned to the Sponsor's project officer as soon as possible. Maintenance of a sample of the test substance is the responsibility of the Study Sponsor, if applicable.

**ARCHIVAL:** The protocol, raw data and the final report will be archived by the Study Sponsor unless different arrangements are made. A copy of the final report will be maintained at Springborn Smithers Laboratories.

### 7.0 REFERENCES

- APHA, AWWA, WPCF. 1992. Standard Methods for the Examination of Water and Wastewater. 18th Edition, Washington, DC.
- OECD, 1984. Guideline for Testing of Chemicals. *Daphnia* sp., Acute Immobilization Test and Reproduction Test. Guideline #202. Adopted 4 April 1984.
- U.S. EPA. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. Ecological Research Series (EPA-660/3-75-009). 61 pp.